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Abstract: We have developed a unique method for mouse transgenesis. The transposase-enhanced pronuclear microinjection (PNI) technique described herein uses the hyperactive piggyBac transposase to insert a large transgene into the mouse genome. This procedure increased transgene integration efficiency by fivefold compared with conventional PNI or intracytoplasmic sperm injection-mediated transgenesis. Our data indicate that the transposase-enhanced PNI technique additionally requires fewer embryos to be microinjected than traditional methods to obtain transgenic animals. This transposase-mediated approach is also very efficient for single-cell embryo cytoplasmic injections, offering an easy-to-implement transgenesis method to the scientific community.

DOI: https://doi.org/10.1073/pnas.1216473109

Originally published at:
Marh, Joel; Stoytcheva, Zoia; Urschitz, Johann; Sugawara, Atsushi; Yamashiro, Hideaki; Owens, Jesse B; Stoytchev, Ilko; Pelczar, Pawel; Yanagimachi, Ryuzo; Moisyadi, Stefan (2012). Hyperactive self-inactivating piggyBac for transposase-enhanced pronuclear microinjection transgenesis. Proceedings of the National Academy of Sciences of the United States of America, 109(47):19184-19189.
DOI: https://doi.org/10.1073/pnas.1216473109

Hyperactive self-inactivating *piggyBac* for transposase-enhanced pronuclear microinjection transgenesis

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Contributed by Ryuzo Yanagimachi, September 21, 2012 (sent for review August 1, 2012)

We have developed a unique method for mouse transgenesis. The transposase-enhanced pronuclear microinjection (PNI) technique described herein uses the hyperactive *piggyBac* transposase to insert a large transgene into the mouse genome. This procedure increased transgene integration efficiency by fivefold compared with conventional PNI or intracytoplasmic sperm injection-mediated transgenesis. Our data indicate that the transposase-enhanced PNI technique additionally requires fewer embryos to be microinjected than traditional methods to obtain transgenic animals. This transposase-mediated approach is also very efficient for single-cell embryo cytoplasmic injections, offering an easy-to-implement transgenesis method to the scientific community.

transgene | genetically modified

Stable transgenesis by integration of exogenous DNA in mammalian zygotes can be achieved using several distinct procedures that have been developed independently over the past 30 y (1). The method most widely used to generate transgenic animals is pronuclear microinjection (PNI), whereby the male pronucleus of a zygote is injected with linear transgene DNA. PNI was originally developed in mice by Gordon et al. (2) and by Brinster and colleagues (3) for rabbits, sheep, and pigs. In subsequent studies, this method was also shown to allow for tissue-specific expression by altering of the transgene's promoter sequences, thus becoming the method of choice for generation of transgenic animal models (3). PNI is a technique requiring considerable embryo manipulation skill, and this passive transgenesis technology relies on the endogenous repair mechanisms of the zygote nucleus for transgene insertion, limiting its effectiveness (4). Moreover, integration of the transgene occurs in a random fashion, usually with multiple concatamerized copies in a single locus after the first cell division of the zygote (5–8). In mice, zygotes transferred to surrogate mothers result in transgenic animals with an efficiency rarely above 3.2% of embryos injected (*ei*), or 25% of animals born (*ab*), with only 70% of the founders being germline transgenic (8, 9). Additionally, a large number of these animals are mosaic, and only about 50% express their transgene at adequate levels (8). In livestock, such as pigs, cattle, and sheep, the high lipid content of the oocyte or of one cell embryo makes it impossible to visualize nuclear structures using a conventional light microscope for implementing PNI (10).

Intracytoplasmic sperm injection-based transgenesis (ICSI-Tr) relies on spermatozoa as DNA vectors (11). Here, spermatozoa are membrane-damaged or demembranated by freeze-thawing or treatment with Triton X-100, and they are then incubated with linear transgene DNA. The exposed perinuclear theca of the sperm head interacts with the transgene and serves as a carrier. This sperm–DNA complex is then injected into the cytoplasm of MII-arrested oocytes (oocytes injected, *oi*), and the transgene is incorporated into the embryonic genome in a passive fashion via the endogenous DNA repair mechanisms (4). As previously observed for PNI, many transgenes integrate as multiple concatamerized vector copies (6, 8, 11, 12). ICSI-Tr, originally developed to generate transgenic mice, has been extended with some success to other species (13). However, the freeze-thawing procedure causes chromosomal damage to some spermatozoa, resulting in increased failure of embryos reaching term (14). As a consequence, the original ICSI-Tr study reported that ∼100 oocytes were needed to obtain 14 liveborn pups, of which 2 were transgenic (11). Such modest levels of efficiency are acceptable for animals that are inexpensive to house and can produce large numbers of offspring in a single litter, but they are prohibitive for many farm animals, such as cattle, that typically produce a single offspring per pregnancy. In nonhuman primates, obtaining oocytes and achieving a successful pregnancy can cost more than $10,000 in Europe; therefore, the lower developmental efficiencies demonstrated by ICSI-Tr make its implementation an expensive proposition (15).

The most efficient method to date is viral transgenesis, initially developed in rodents and then extended to farm animals (16, 17). Here, disarmed lentiviral vectors are used to insert transgenes actively at the single-cell embryo stage (16). Using this approach, 23% of the micromanipulated oocytes result in transgenic animals, with an overall percentage of 80% for *ab*. Among the disadvantages of this technique are the high embryo lethality (27% survival percentage) and relatively small-sized cargo capacity of 9.5 kb due to the limited amount of DNA that can be packaged within the viral particle (16). Furthermore, specialized containment facilities for lentiviral production are required, making it prohibitive for many laboratories to use virus-based transgenesis. Finally, the potential of recombinant events between the viral vector and endogenous host viruses, leading to the generation of new and more potent pathogenic agents, is of concern (1). Therefore, the development of new transgenesis methods that are safer and more efficient and are not hampered by transgene size limitations would be desirable.

We have recently developed an improved ICSI-Tr method that uses the *piggyBac* transposase for efficient gene transfer into the mouse genome (18, 19). We have engineered unique *piggyBac* plasmid vectors that contain the transposon cargo and the *piggyBac* transposase gene on a single-helper independent plasmid (p*m*GENIE-3). Moreover, the vector design facilitates self-inactivation of the transposase on excision of the transposon. These features were shown to reduce the construct's genotoxic potential while simultaneously improving transposon integration efficiency. We have demonstrated that this design allowed us to reduce the number of transgene integrations markedly, often to one per genome (19). Here, we report the development of a series of unique hyperactive plasmid vectors (p*mhy*GENIE-3) based on a previously described hyperactive mammalian codon-optimized *piggyBac* transposase variant (20). We have

Author contributions: S.M. designed research; J.M., Z.S., J.U., A.S., H.Y., J.B.O., I.S., P.P., and S.M. performed research; R.Y. and S.M. analyzed data; and Z.S., J.U., J.B.O., P.P., R.Y., and S.M. wrote the paper.

The authors declare no conflict of interest.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1216473109/-/DCSupplemental.

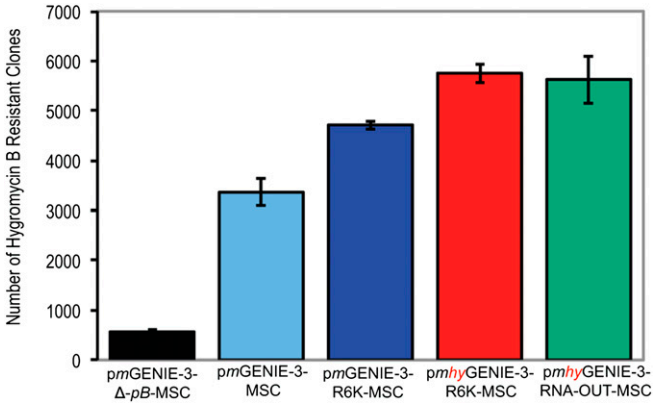

Fig. 1. Comparison of plasmid transfection efficiencies in HEK293T cells. Each of the improvements to the vectors either increases the transfection efficiency or adds a significant feature without reducing the efficiency (*pB*, *piggyBac*).

evaluated the transgenesis efficiency of these constructs in combination with standard transgenesis techniques, such as PNI and ICSI. We have achieved transgene integration without having to pretreat the spermatozoa during ICSI-Tr, thus facilitating embryo development percentages similar to traditional nontransgenic ICSI methods (21). More importantly we have used the p*mhy*GENIE system to develop a unique transgenesis technique: transposase-enhanced pronuclear microinjection (te-PNI). When using p*mhy*GENIE-3 plasmids in combination with te-PNI, we achieved a far greater transgenic percentage than all other methods currently used in mice. Furthermore, to develop a transgenesis technique for application in livestock that facilitates improved development of microinjected zygotes to live offspring, we explored direct cytoplasmic injections (CTIs) of the p*mhy*GENIE-3 constructs into one-cell mouse embryos. This technique not only facilitated transgene integration, but the observed transgenesis percentages using this method were significantly higher than for those for conventional PNI.

Results

Cell Culture Experiments with Improved p*m*GENIE Plasmids. We have created unique self-inactivating hyperactive *piggyBac* transposase-based plasmids (p*mhy*GENIE-3) for animal transgenesis. Building on our previously published p*m*GENIE constructs (19), we have further improved these vectors to make them more effective for integrative gene transfer. As a first step, we introduced an SV40 enhancer sequence, which has been reported to increase the transfer of plasmids from the cytoplasm into the nucleus (22–24). Using p*m*GENIE-3-R6K in in vitro experiments, we noted a 40% increase in colony formation under identical transformation and selection conditions compared with the original p*m*GENIE-3 construct (Fig. 1). In a second modification, we replaced the mouse codon-biased *piggyBac* with the hyperactive *piggyBac* gene (p*mhy*GENIE-3-R6K) (20). We observed improvements to the transfection efficiency above those achieved with p*m*GENIE-3-R6K. As a final improvement, we engineered the vectors to feature an antibiotic-free selection (*pmhyGENIE-3-RNA-OUT*) in bacteria to conform to regulatory recommendations for the elimination of antibiotic resistance markers during in vivo transfection and transgenesis (25). A comparison of colony counts between the original p*m*GENIE-3 plasmid and the newly developed hyperactive plasmid constructs, as depicted in Fig. 1, demonstrates that each of the improvements to the vectors either increases the transfection efficiency (p*m*GENIE-3-R6K and p*mhy*GENIE-3-R6K) or adds a significant feature without reducing its efficiency (*pmhyGENIE-3-RNA-OUT*) (Table S1).

Transgenesis via te-PNI. To establish a baseline for PNI efficiency in our hands, we performed a set of PNI injections with a large-bore pipette (2 μm) in combination with a Piezo actuator and with a linear transgene matching the size of the p*mhy*GENIE-3 plasmid mammalian selection cassette (MSC) (Fig. 2 and Movie S1). The movie demonstrates the low Piezo actuator pulses necessary to penetrate the zona pellucida and the two membrane layers of the embryo to reach the male pronucleus. Using this traditional experimental setup for transgenesis, we achieved an efficiency of 2.0% for *ei* (Table 1), a result in the range of published PNI data from several laboratories worldwide (9, 26). We next injected the nonhyperactive p*m*GENIE-3-R6K-MSC into paternal pronuclei by te-PNI (Table 2). Transgenic percentages for embryos micromanipulated ranged from 13.4–22.5% for *ei* and from 58.0–60.5% for *ab*, a significant increase over conventional PNI, which has percentages of around 3.2% for *ei* and 24.0% for *ab* (9, 26).

In subsequent transgenesis experiments, we switched to the hyperactive p*mhy*GENIE-3-RNA-OUT-MSC construct, because this construct allowed us to perform transgenesis experiments without antibiotic selection (Fig. 1 and Table 3).

As an initial step, we titrated plasmid concentrations ranging from 0.25 to 40.0 ng/μL to obtain the optimal result for a given plasmid concentration. The results, shown in Table 3, indicate percentages comparable to PNI at lower concentrations, whereas the highest concentration, 40.0 ng/μL, was lethal, and no live pups were obtained presumably because the abundant amount of DNA exerted toxic effects. The concentrations of 7.5 and 10.0 ng/μL were most effective for transgenic animal production, and we subsequently performed a large-scale microinjection series with 10.0-ng/μL injections. We obtained average percentages of 25.9% for *ei* and 59.7% for *ab* (Table 3).

Traditional PNI with p*m*GENIE Plasmids. To compare te-PNI directly with traditional PNI using our circular DNA constructs, we injected p*mhy*GENIE-3-RNA-OUT-MSC into the paternal pronuclei of embryos via PNI, with narrow-bore pointed-tip pipettes and no Piezo actuator. At 10-ng/μL plasmid concentrations, the transgenic percentages for *ei* and *ab* were 2.1% and 100%, respectively, showing that although the construct did perform well in terms of generating transgenic mice, using it in the context of classic PNI caused significant embryo lethality. Decreasing the DNA concentration to 2 ng/μL did improve embryo survival percentages; however, transgene integration percentages dropped to levels similar to those observed with PNI of standard linear DNA (2.3% *ei* and 17.9% *ab*) (Table 3). All the transgenic founder pups demonstrated variegated gene expression. These results clearly demonstrate that the combination of p*m*GENIE plasmids and improvements to PNI described above is necessary to obtain increased transgenesis percentages.

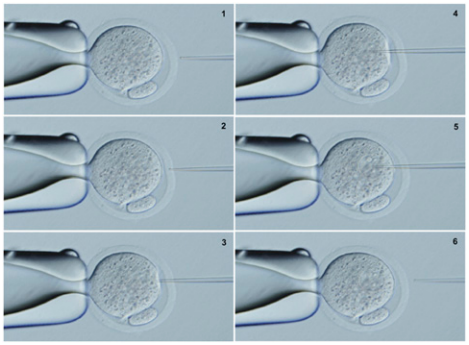

Fig. 2. Zona-drilling before te-PNI. The tip of the injection pipette is brought into contact with the zona pellucida (1) of the one-cell embryo, and a Piezo pulse is applied (2), allowing penetration of the pipette into the cytoplasm (3). A second Piezo pulse is applied to allow pipette penetration through the pronuclear membrane (4). The pronucleus is extended during plasmid injection (5), and ~130 copies of GENIE-3 plasmids are delivered into it. The pipette is withdrawn (6). At no time during the te-PNI process is the embryo compressed. (Magnification, 30×.)

Table 1. te-PNI done with a linear transgene containing a CAG-driven EGFP gene, SV40 promoter-driven hygromycin gene for selection in mammalian cells, and a bacterially expressed kanamycin gene (7,244 bp)

No. of *ei* (replicates)	Linear transgene containing CAG-EGFP + hygromycin + kanamycin (7,244 bp), ng/μL	No. of embryos survived (% injected, *ei*)	No. of two-cell stage embryos transferred (% injected, *ei*)	No. of surrogates used for transfer	No. of liveborn pups (% injected, *ei*) [% transferred]	No. of EGFP-positive pups (% injected, *ei*) [% transferred] {% born, *ab*}
110 (1)	2.0	106 (96.4)	106 (96.4)	7	27 (24.5) [25.5]	2 (1.8) [1.9] {7.4}

Transgenesis via CTI of p*mhy*GENIE-3-RNA-OUT-MSC Plasmid. To develop a more efficient transgenesis approach for livestock, in which the high lipid content of the oocyte impedes visualization of the nuclear structures by conventional light microscopic techniques, we directly injected p*mhy*GENIE-3-RNA-OUT-MSC into the cytoplasm of one-cell embryos of B6D2F2 zygotes. Again, we titrated plasmid concentrations in 10-ng increments, starting at the optimal concentration for te-PNI of 10.0 ng/μL and increasing the DNA concentration up to 50.0 ng/μL (Table 4). We obtained transgenic mouse pups at all plasmid concentrations with efficiencies comparable to or higher than previously published PNI percentages (26). The best results were achieved at a plasmid concentration of 10.0 ng/μL, resulting in average transgenic percentages of 18.5% for *ei* and 34.5% for *ab* (Table 4).

ICSI-Tr. To avoid potential side effects caused by the pretreatment of spermatozoa, we performed ICSI-Tr with p*mhy*GENIE-3-RNA-OUT-MSC and fresh sperm. The percentages for *oi* were low and sometimes did not result in transgenic pups at all. Using spermatozoa treated with 10 mM NaOH as a control, we obtained percentages similar to those previously reported (19) (Table 5). With fresh sperm and the lowest plasmid concentration of 1.0 ng/μL, we observed percentages equivalent to those obtained with NaOH treatment. Higher plasmid concentrations resulted in a decrease in efficiency.

Monoclonal Antibody Studies to Assess *piggyBac* Localization Expressed from p*mhy*GENIE Plasmids. Previously, we used a polyclonal antibody to assess *piggyBac* localization expressed from newly developed constructs synthesized with chimeric *piggyBac* transposases (19). To gain an understanding of the functional competence of the newly developed constructs synthesized with chimeric *piggyBac* transposases, we performed immunolocalization with a newly produced monoclonal antibody against the *piggyBac* protein. The data obtained demonstrated expression patterns of nuclear localization for the de novo synthesized transposase protein in mouse embryos, without nonspecific binding to endogenous mouse proteins (Fig. 3). Immunolocalization persisted up to the blastocyst stage.

Single-Copy Transgene Integration with p*mhy*GENIE-3-RNA-OUT-MSC. Commonly used transgenesis techniques, such as PNI or ICSI-Tr, often result in multicopy concatamerized transgene arrays that frequently influence transgene expression (27). This concatamerized transgene phenomenon is believed to be due to the ligation of linear transgenes in a head-to-tail orientation by the homologous recombination mechanism of the oocyte DNA repair machinery. The subsequent integration into the host genome is then mediated by nonhomologous recombination (5, 7, 28, 29). Gene transfer by the *piggyBac* transposase seems to avoid such concatamers. During *piggyBac* transposition, a single transposon is excised from the plasmid to form a synaptic complex. The cut-and-paste mechanism of type II transposases appears to ensure that only individual transgenes excised from the plasmid participate in transposition (19). We used Southern blotting to evaluate whether the methods described here show a propensity for generating concatameric insertions. As shown in Fig. 4, all zero filial (F_0) animals produced by fresh sperm ICSI-Tr, te-PNI, or CTI carried single-copy insertions, as additionally verified by genomic site of insertion analysis (Fig. S3). All microinjection techniques resulted in one to four insertions per animal. As we described previously, this transgene copy range does not appear to cause any detrimental mutations to F_0 animals (19). The data in Table S2 additionally demonstrate that all 18 F_0 animals tested for te-PNI were germline transgenic, giving rise to fully transgenic first filial (F_1) generations. Of the 12 CTI animals tested, 10 were germline transgenic, whereas for ICSI-Tr–mediated transgenesis with fresh sperm, all four F_0 animals tested passed on their transgene through the germline to the F_1 generation.

Genomic Site of Insertion Analysis. We previously reported transposon insertion site analysis for ICSI-Tr–generated transgenic animals, which was done with Vectorette PCR and inverse PCR (19). For the current experiments, a nonrestrictive linear amplification-mediated (nrLAM) PCR approach was used for the identification of transpositionally generated sites of insertion for te-PNI– and CTI-generated transgenic F_0 animals. Two insertion sites for te-PNI animals 2 and 3 and an additional two insertion sites for CTI animals 1 and 2, depicted in Fig. 4, were recovered (Fig. S3). Therefore, for each transgenesis approach used, the genomic insertion of the transgene found in the transposon is transpositionally generated.

Discussion

Transgenic animals were originally developed in rodent research to model human diseases, such as cancer, heart disease, and neurodegenerative disorders (e.g., Huntington disease, Alzheimer's disease) (30). The insights gained with these animals elucidated gene functions at the molecular, cellular, and systems levels and have helped unravel mechanisms in physiology and pathology. Knowledge acquired in small rodents has been applied to large-animal disease models and has paved the way for drug and biological therapy in medicine (31, 32). Recently, there has been an interest in the transgenesis of ruminants because of their potential

Table 2. te-PNI performed with p*m*GENIE-3-R6K-MSC

No. of *ei* (replicates)	p*m*GENIE-3-R6K-MSC, ng/μL (*final fg concentration*)	No. of embryos survived (% injected, *ei*)	No. of two-cell stage embryos transferred (% injected, *ei*)	No. of surrogates used for transfer	No. of liveborn pups (% injected, *ei*) [% transferred]	No. of EGFP-positive pups (% injected, *ei*) [% transferred] {% born, *ab*}
108 (3)	H_2O control	108 (100.0)	108 (100.0)	6	62 (57.4) [57.4]	0
134 (2)	3.5 (*0.71*)	123 (92.0)	70 (52.2)	6	31 (23.1) [42.3]	18 (13.4) [25.7] {58.0}
306 (6)	7.5 (*1.53*)	292 (95.4)	263 (85.9)	15	114 (37.3) [43.3]	69 (22.5) [26.2] {60.5}

Table 3. Summary of te-PNI performed with the p*mhy*GENIE-3-RNA-OUT-MSC plasmid

No. of *ei* (replicates)	p*mhy*GENIE-3-RNA-OUT-MSC, ng/μL (*final fg concentration*)	No. of embryos survived (% injected, *ei*)	No. of one-cell or two-cell stage embryos transferred (% injected, *ei*)	No. of surrogates used for transfer	No. of liveborn pups (% injected, *ei*) [% transferred]	No. of EGFP-positive pups (% injected, *ei*) [% transferred] {% born, *ab*}
15 (1)	0.25 (*0.05*)	13 (86.7)	13* (86.7)	1	6 (40.0) [46.2]	0 (0.0) [0.0] {0.0}
78 (1)	0.5 (*0.10*)	73 (93.6)	73* (93.6)	4	29 (37.2) [39.7]	2 (2.5) [2.7] {6.9}
70 (1)	1.0 (*0.20*)	60 (85.7)	60* (85.7)	3	20 (28.5) [33.3]	3 (4.3) [5.0] {15.0}
60 (1)	2.0 (*0.40*)	57 (95.0)	57* (95.0)	3	18 (30.0) [31.5]	3 (5.0) [5.3] {16.6}
65 (1)	4.0 (*0.80*)	62 (95.4)	62* (95.4)	3	8 (37.0) [12.9]	2 (3.1) [3.2] {25.0}
137 (3)	7.5 (*1.53*)	113 (82.5)	113* (82.5)	7	33 (24.1) [29.2]	15 (10.9) [13.3] {45.5}
88 (1)	10.0 (*2.04*)	78 (88.6)	78* (88.6)	6	20 (22.7) [25.6]	19 (21.6) [24.4] {95.0}
70 (2)	20.0 (*4.08*)	63 (90.0)	28† (40.0)	2	4 (5.7) [14.3]	0 (0.0) [0.0] {0.0}
60 (1)	40.0 (*8.16*)	53 (88.3)	19† (31.7)	2	0 (0.0) [0.0]	0 (0.0) [0.0] {0.0}
799 (14)	10.0 (*2.04*)	764 (95.6)	700† (87.6)	45	347 (43.4) [49.6]	207 (25.9) [29.6] {59.7}
562 (4)	2.0 (*0.40*)	362 (64.4)	362*,‡ (64.4)	17	74 (13.2) [20.4]	13 (2.3) [3.6] {17.6}
91 (1)	10.0 (*2.04*)	66 (72.5)	66*,‡ (72.5)	3	2 (2.1) [3.0]	2 (2.1) [3.0] {100}

*Embryos transferred at the one-cell stage.
†Embryos transferred at the two-cell stage.
‡Injections performed by traditional PNI with a 0.1- to 0.2-μm internal diameter pipette and the help of an automated microinjector.

use as bioreactors for the production of valuable biomolecules (33). However, the application of PNI to large animals is impeded by low efficiency, concatamerized transgene insertion into the genome, and unpredictable levels and patterns of transgene expression.

Only a few species are amenable to the micromanipulations necessary for efficient ICSI-Tr. For example, ICSI-Tr is inefficient in pigs, and therefore appears unsuitable for routine transgenic production (34–36). Lentiviral-mediated transgenesis, on the other hand, is very efficient in delivering the transgene to the host genome but results in multicopy insertions; as a consequence, only a few micromanipulated embryos reach term, probably due to lethal mutagenesis. Of the resulting live births however, more than 80% are transgenic (16). High percentages of embryo lethality are acceptable in mice but are more problematic in species in which birth numbers are limited to one or two offspring per pregnancy.

We have therefore developed a unique transgenesis method (te-PNI) that is at least fivefold more efficient in mice than traditional PNI (for *ei*). te-PNI is based on using *piggyBac* transposase vectors, larger diameter injection pipettes, and a Piezo actuator. Transgenesis percentages with p*mhy*GENIE-3-RNA-OUT-MSC at a concentration of 10 ng/μL (25.9% for *ei* and 59.7% of *ab,* Table 3) demonstrate that in terms of efficiency, te-PNI is equivalent to lentiviral transgenesis. Although variegated expression of the transgene was fairly high for F_0 animals at ~70%, all transgenic offspring (F_1) produced by these founders

Table 4. CTI performed with a 2-μm internal diameter pipette and a Piezo actuator

No. of *ei* (replicates)	p*mhy*GENIE-3-RNA-OUT-MSC, ng/μL (*final fg concentration*)	No. of embryos survived (% injected, *ei*)	No. of two-cell stage embryos transferred (% injected, *ei*)	No. of surrogates used for transfer	No. of liveborn pups (% injected, *ei*) [% transferred]	No. of EGFP-positive pups (% injected, *ei*) [% transferred] {% born, *ab*}
156 (5)	10.0 (*2.04*)	127 (81.4)	126 (80.8)	9	84 (53.8) [66.6]	29 (18.5) [23.0] {34.5}
83 (2)	25.0 (*5.10*)	77 (92.7)	65 (78.3)	5	33 (39.8) [50.8]	10 (12.0) [15.4] {30.3}
40 (1)	35.0 (*7.14*)	40 (100.0)	40 (100.0)	3	17 (35.0) [35.0]	1 (2.5) [2.5] {7.1}
30 (1)	40.0 (*8.16*)	30 (100.0)	30 (100.0)	2	13 (30.0) [30.0]	3 (10.0) [10.0] {10.0}
25 (1)	50.0 (*10.20*)	25 (100.0)	25 (100.0)	2	14 (20.0) [20.0]	3 (12.0) [12.0] {60.0}

Table 5. Summary of ICSI-Tr injections performed with a 7-μm internal diameter pipette and a Piezo actuator

No. of *oi* (replicates)	Sperm treatment	p*mhy*GENIE-3-RNA-OUT-MSC, ng/μL (*final fg concentration*)	No. of two-cell stage embryos transferred (% injected, *oi*)	No. of surrogates used for transfer	No. of liveborn pups (% injected, *oi*) [% transferred]	No. of EGFP-positive pups (% injected, *oi*) [% transferred] {% born, *ab*}
75 (1)	10 mM NaOH	0.5 (*0.10*)	61 (81.3)	5	13 (17.3) [21.3]	4 (5.3) [6.6] {30.8}
99 (1)	10 mM NaOH	1.0 (*0.20*)	68 (68.7)	6	10 (10.1) [14.7]	4 (4.0) [5.9] {40.0}
57 (3)	Fresh	1.0 (*0.20*)	52 (91.2)	3	16 (28.0) [30.8]	3 (5.3) [5.8] {18.8}
20 (1)	Fresh	2.0 (*0.40*)	18 (90.0)	2	3 (15.0) [16.6]	0 (0.0) [0.0] {0.0}
140 (3)	Fresh	5.0 (*1.02*)	92 (65.7)	10	17 (12.1) [18.5]	1 (0.7) [1.1] {5.9}
140 (2)	Fresh	10.0 (*2.04*)	105 (75.0)	7	10 (7.1) [9.5]	1 (0.7) [1.0] {10.0}
220 (3)	Fresh	15.0 (*3.06*)	84 (38.2)	7	0 (0.0) [0.0]	0 (0.0) [0.0] {0.0}
92 (4)	Fresh	25.0 (*5.10*)	41 (44.6)	5	1 (1.1) [2.4]	0 (0.0) [0.0] {0.0}
113 (2)	Fresh	50.0 (*10.20*)	54 (47.8)	5	3 (2.7) [5.6]	2 (1.8) [3.7] {66.6}
113 (2)	Fresh	100.0 (*20.40*)	29 (25.7)	3	1 (0.9) [3.4]	0 (0.0) [0.0] {0.0}
200 (2)	Fresh	200.0 (*40.80*)	9 (4.5)	2	1 (0.5) [11.1]	1 (0.5) [11.1] {100.0}

displayed transpositionally generated uniform and robust transgene expression. All experiments were performed in mice; however, we believe that the techniques described herein can be easily adapted for other species. Future research in livestock will determine if the timing of transcription initiation from plasmid DNA in large-animal zygotes differs from that in mice because that may influence the effectiveness of this approach for these animals.

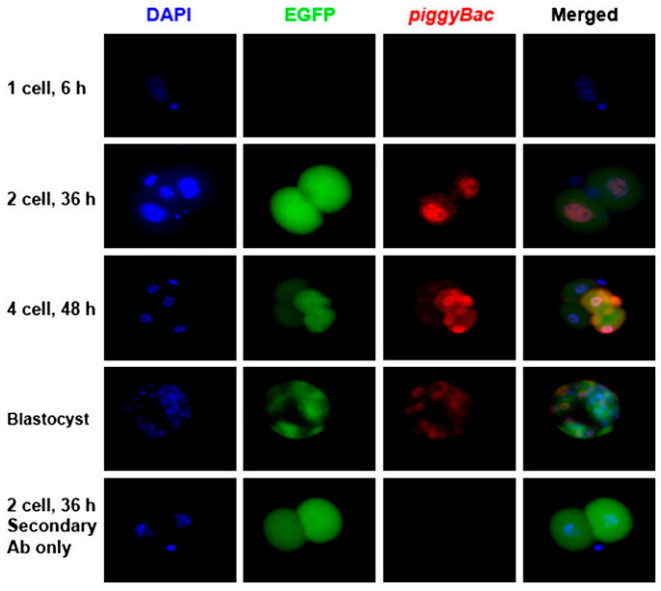

Fig. 3. Time course of *piggyBac* and EGFP protein expression in transgenic embryos generated by te-PNI. Expression of *piggyBac* transposase (*pBt*) was visualized by immunofluorescence using a monoclonal antibody for the *pBt* protein and is detectable above background levels at 36 h, corresponding to the detection of EGFP transgene expression. DAPI was used to visualize nuclei.

CTI of p*m*GENIE plasmids was approximately fourfold more efficient than traditional PNI. This approach might therefore provide a valuable tool for transgenesis in species in which visualization of the pronuclei is difficult due to high lipid content in the oocyte or zygote. Additionally, the injection of DNA into the cytoplasm of zygotes is considerably less cumbersome compared with other transgenesis micromanipulation techniques, and can therefore be applied without the technical expertise necessary for PNI.

By using ICSI-Tr implemented with fresh sperm for the fertilization of oocytes, we aimed to avoid potential detrimental effects to the spermatozoa caused by the treatment necessary to adhere DNA to their surface. The highest transgenesis percentages were attained using the lowest plasmid concentration and were comparable to those obtained with NaOH-treated sperm. Higher plasmid concentrations resulted in lower efficiencies, potentially due to the possibility that these concentrations prove toxic to the embryos. Another factor influencing ICSI-Tr efficiency is that the injections of plasmids during fertilization are performed at a very early developmental stage, whereas injections for te-PNI and CTIs take place at the later developmental stage of two pronuclei. This difference of 6 h may be critical for the persistence of episomal plasmids that may be exposed to nucleases present in the cytoplasm before the initiation of transcription at the two-cell stage. Increasing the plasmid concentration increases the number of vectors available at this developmental time point but appears to have a negative effect on embryo survival.

Comparing the efficiency of different transgenesis techniques reported by individual research groups can be challenging. For example, the ratio of total animals born (*tab*)/animals transgenic (*at*) has been used previously. Two prior publications on transposase-based transgenesis stated *tab*/*at* ratios of 34.8% (37) and 37% (38), but the number of micromanipulated embryos (*ei*) was omitted. Using this approach, we achieved efficiencies as high as 95% in one experiment, with an average of almost 60% for 10-ng/μL DNA plasmid injections (Table 3). However, because transgenesis techniques suffer from poor postembryonic development, this ratio does not necessarily reflect the overall efficiency of a particular technique. We believe that a more accurate reflection of the effectiveness of a method is reporting transgenic

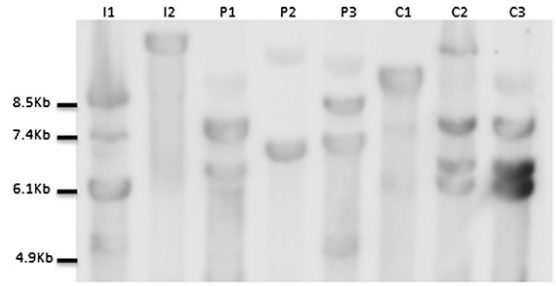

Fig. 4. Southern blot analysis of genomic DNA from p*mhy*GENIE-3-RNA-OUT transgenic F_0 mice. I lanes are ICSI-Tr–generated transgenics, P lanes represent te-PNI transgenics, and C lanes denote CTI-produced transgenics.

animals born as a ratio of total embryos micromanipulated; we report here a transgenesis efficiency for te-PNI of 25.9%. This percentage represents a significant increase in efficiency, because traditional PNI does not exceed 5% for *ei/at*. Unfortunately, a direct comparison with the two previous reports mentioned above is not possible. Additionally, other laboratories used transposase mRNA for transgenesis (39–41). Although this approach is feasible, we believe that te-PNI and CTI provide an improved approach because they do not require the manipulation of easily degradable RNA molecules. In summary, the methods described herein facilitate the production of transgenic animals with significantly increased transgenic percentages that are comparable to lentiviral methods but simpler to implement.

Materials and Methods

Here, we provide a brief summary of the applied methods. A detailed description is provided in *SI Materials and Methods*.

Plasmid Development. The pmGENIE plasmids were constructed by basic molecular biology methods as described previously (19). The GATEWAY recombineering method of Life Technologies was used for the construction of the final transposon-bearing p*mhy*GENIE plasmids by the use of LR Clonase enzyme (Life Technologies).

Cell Transfections. Cell culture and transfections were performed as described previously (19).

Southern Blot Analysis. Southern blot analysis was performed as described previously (19).

Transposon Insertion Sites. Genomic transpositional insertion sites were identified by nrLAM-PCR, which has been described previously (42, 43).

Immunohistochemistry. After fixation and removal of the zona pellucida, oocytes were incubated with primary *piggyBac* antibody, followed by secondary antibody staining as described previously (19).

Transgenesis. The procedures were performed as previously described with only minor modifications (12, 19, 44).

ACKNOWLEDGMENTS. We thank the Sanger Institute (Cambridge, England) for the mouse codon-optimized hyperactive *piggyBac* plasmid. This work was supported by National Institutes of Health Grants 5P20RR024206 and R01 GM083158-01A1 (to S.M.).

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